

the enzymes of this class can also be made to catalyze the invader-directed cleavage of the present invention, albeit not as efficiently as the Cleavase® enzymes.

Cleavase® BN nuclease and Cleavase® A/G nuclease were tested along side three different thermostable DNA polymerases: *Thermus aquaticus* DNA polymerase (Promega), *Thermus thermophilus* and *Thermus flavus* DNA polymerases (Epicentre). The enzyme mixtures used in the reactions shown in lanes 1-11 of Figure 38 contained the following, each in a volume of 5 µl: Lane 1: 20 mM MOPS (pH 7.5) with 0.1% each of Tween 20 and NP-40, 4 mM MnCl₂, 100 mM KCl; Lane 2: 25 ng of Cleavase® BN nuclease in the same solution described for lane 1; Lane 3: 2.25 µl of Cleavase® A/G nuclease extract (prepared as described in Example 2), in the same solution described for lane 1; Lane 4: 2.25 µl of Cleavase® A/G nuclease extract in 20 mM Tris-Cl, (pH 8.5), 4 mM MgCl₂ and 100 mM KCl; Lane 5: 11.25 polymerase units of *Taq* DNA polymerase in the same buffer described for lane 4; Lane 6: 11.25 polymerase units of *Tth* DNA polymerase in the same buffer described for lane 1; Lane 7: 11.25 polymerase units of *Tth* DNA polymerase in a 2X concentration of the buffer supplied by the manufacturer, supplemented with 4 mM MnCl₂; Lane 8: 11.25 polymerase units of *Tth* DNA polymerase in a 2X concentration of the buffer supplied by the manufacturer, supplemented with 4 mM MgCl₂; Lane 9: 2.25 polymerase units of *Tfl* DNA polymerase in the same buffer described for lane 1; Lane 10: 2.25 polymerase units of *Tfl* polymerase in a 2X concentration of the buffer supplied by the manufacturer, supplemented with 4 mM MnCl₂; Lane 11: 2.25 polymerase units of *Tfl* DNA polymerase in a 2X concentration of the buffer supplied by the manufacturer, supplemented with 4 mM MgCl₂.

Sufficient target DNA, probe and invader for all 11 reactions was combined into a master mix. This mix contained 550 fmoles of single-stranded M13mp19 target DNA, 550 pmoles of the invader oligonucleotide (SEQ ID NO:46) and 55 pmoles of the probe oligonucleotide (SEQ ID NO:43), each as depicted in Figure 32c, in 55 µl of distilled water. Five µl of the DNA mixture was dispensed into each of 11 labeled tubes and overlaid with a drop of ChillOut® evaporation barrier (MJ Research). The

reactions were brought to 63°C and cleavage was started by the addition of 5 µl of the appropriate enzyme mixture. The reaction mixtures were then incubated at 63°C temperature for 15 minutes. The reactions were stopped by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. Samples were heated to 90°C for 1 minute immediately before electrophoresis through a 20% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris-Borate (pH 8.3), 1.4 mM EDTA. Following electrophoresis, the products of the reactions were visualized by the use of an Hitachi FMBIO fluorescence imager, and the results are displayed in Figure 38. Examination of the results shown in Figure 38 demonstrates that all of the 5' nucleases tested have the ability to catalyze invader-directed cleavage in at least one of the buffer systems tested. Although not optimized here, these cleavage agents are suitable for use in the methods of the present invention.

EXAMPLE 18

The Invader-Directed Cleavage Assay Can Detect Single Base Differences In Target Nucleic Acid Sequences

The ability of the invader-directed cleavage assay to detect single base mismatch mutations was examined. Two target nucleic acid sequences containing Cleavase® enzyme-resistant phosphorothioate backbones were chemically synthesized and purified by polyacrylamide gel electrophoresis. Targets comprising phosphorothioate backbones were used to prevent exonucleolytic nibbling of the target when duplexed with an oligonucleotide. A target oligonucleotide, which provides a target sequence that is completely complementary to the invader oligonucleotide (SEQ ID NO:46) and the probe oligonucleotide (SEQ ID NO:43), contained the following sequence: 5'-CCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTTCGCCGGC-3' (SEQ ID NO:47). A second target sequence containing a single base change relative to SEQ ID NO:47 was synthesized: 5'-CCTTTCGCTCTCTTCCCTTCCTTTCTCGCCACGTTTCGCCGGC-3 (SEQ ID NO:48; the single base change relative to SEQ ID

NO:47 is shown using bold and underlined type). The consequent mismatch occurs within the "Z" region of the target as represented in Figure 29.

To discriminate between two target sequences which differ by the presence of a single mismatch), invader-directed cleavage reactions were conducted using two different reaction temperatures (55°C and 60°C). Mixtures containing 200 fmoles of either SEQ ID NO:47 or SEQ ID NO:48, 3 pmoles of fluorescein-labelled probe oligonucleotide (SEQ ID NO:43), 7.7 pmoles of invader oligonucleotide (SEQ ID NO:46) and 2 µl of Cleavase® A/G nuclease extract (prepared as described in Example 2) in 9 µl of 10 mM MOPS (pH 7.4) with 50 mM KCl were assembled, covered with a drop of ChillOut® evaporation barrier (MJ Research) and brought to the appropriate reaction temperature. The cleavage reactions were initiated by the addition of 1 µl of 20 mM MgCl₂. After 30 minutes at either 55°C or 60°C, 10 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes was added to stop the reactions. The reaction mixtures were then heated to 90°C for one minute prior to loading 4 µl onto 20% denaturing polyacrylamide gels. The resolved reaction products were visualized using a Hitachi FMBIO fluorescence imager. The resulting image is shown in Figure 39.

In Figure 39, lanes 1 and 2 show the products from reactions conducted at 55°C; lanes 3 and 4 show the products from reactions conducted at 60°C. Lanes 1 and 3 contained products from reactions containing SEQ ID NO:47 (perfect match to probe) as the target. Lanes 2 and 4 contained products from reactions containing SEQ ID NO:48 (single base mis-match with probe) as the target. The target that does not have a perfect hybridization match (*i.e.*, complete complementarity) with the probe will not bind as strongly, *i.e.*, the T_m of that duplex will be lower than the T_m of the same region if perfectly matched. The results presented here show that reaction conditions can be varied to either accommodate the mis-match (*e.g.*, by lowering the temperature of the reaction) or to exclude the binding of the mis-matched sequence (*e.g.*, by raising the reaction temperature).

The results shown in Figure 39 demonstrate that the specific cleavage event which occurs in invader-directed cleavage reactions can be eliminated by the presence